Apple phenolics and their contribution to enzymatic browning reactions

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Abstract

Chlorogenic acid, epicatechin, procyanidin B2 and C1 were isolated from apple skin. These compounds as well as quercetine and phloretine glycosides isolated from apples were studied individually and as mixtures for their participation in the enzymatic browning reactions. The importance of quercetine glycosides and the synergistic effect of phloridzin and phloretine xyloglucoside with chlorogenic acid and flavans in the browning reaction are reported.

Key words: apple, phenolics, browning

INTRODUCTION

The color changes of fruits and vegetables during processing or storage are of great interest in food technology. The brown color development in bruised apple fruit, processed apple juice or wine is related primarily to phenolic

oxidation, the process which may occur nonenzymatically or which is catalysed by o-diphenol oxidase (ODP) (Whitaker 1972). The intensity of browning has been determined by the amount of the phenolic substrate as well as by ODP activity (Weurman and Swain 1955, Walker 1962, Harel et al. 1964, 1966, Marcheix 1970, Vamos-Vigyazo et al. 1976, 1977). The most prevalent substrates in apples for this enzymatic reaction are chlorogenic acid and epicatechin (Walker 1962, 1964, Harel et al. 1964, Monties 1966, Marcheix 1970), with epicatechin being more readily oxidised than chlorogenic acid (Siegelman 1955, Lea 1978, Vamos-Vigyazo and Gajzago 1978). However, apple phenolics also consist of other phenolics such as procyanidins, flavonol and dihydrochalcone glycosides (Lea and Timberlake 1974, Whitting and Coggins 1975, Lea and Beech 1978, Lea 1984), the contribution of which to the browning reaction has not been fully documented.

The flavonol-ODP relationships in respect to browning reactions were closely studied by Shannon and Pratt (1967). Although it was shown that dihydroquercetin was a ODP substrate, fisetin and rhamnetin were found to be inhibitors, whereas quercetin, quercitrin and rutin did not participate in the browning reactions. No reasonable explanation was found to interpret these differences. Moreover, it was shown that neither dihydroquercetin nor fisetin produced any synergistic effect on the rate of browning when present in the solution together with chlorogenic acid.

Durkee and Poapst (1965), who studied the role of phloridzin in the browning reactions reported that phloridzin was not a ODP substrate, but there has been no information available on phloretine xyloglucoside. Since these two dihydrochalcone glycosides are important phenolic components of apples (Oleszek et al. 1988), the knowledge of their participation in enzymatic oxidation is desirable. In this paper, we report the role of these compounds as well as of other new compounds isolated from apples in color development in model systems to better understand the enzymatic browning in apples.

MATERIALS AND METHODS

Apples. Rhode Island Greening apples grown at the New York State Agricultural Experiment Station orchards at Geneva, New York during the 1986 season were used in this study.

Isolation of chlorogenic acid. The same extraction procedure as previously described (Oleszek et al. 1988) was followed. The methanol extract of the apple skin (300 g) was washed with hexane to remove lipids and pigments. The extract was redissolved in water, adjusted to pH 7.0, loaded onto C_{18} column (Waters Associates, 55-105 μ m, 50 g) and then eluted with 1 dm³ of distilled

water. The eluate was adjusted to pH 2.5 with HCl and then phenolic acids were extracted with ethyl acetate. After removal of the solvent, a chromatographically (TLC) homogeneous sample (260 mg) was obtained as a non-crystalline glass.

Fractionation of procyanidins. The phenolics adsorbed on the C_{18} column were eluted with methanol and then separated into several fractions by preparative HPLC as previously described (Oleszek et al. 1988). The first fraction which was eluted with 8% acetonitrile in 5% acetic acid and contained catechins and procyanidins (2.5 g), was loaded onto a column of Sephadex LH 20 (30 cm $\times$ 2.5 cm) previously equilibrated with ethanol. The column was subsequently washed (1 cm³/min) with ethanol (0.5 dm³), ethanol:methanol 1:1 v/v (0.5 dm³) and methanol (0.5 dm³) to yield four different fractions as monitored by UV detector.

Thin layer chromatography (TLC). TLC was performed on Silica gel (60, F-254, Merck) with toluene-chloroform-formic acid (3:6:1, v/v) as a mobile phase. Spots were made visible by spraying with a Folin-Ciocalteau spray reagent (Fisher Scientific).

Analysis of phenolics by analytical HPLC and FAB-MS. Analytical HPLC and FAB-MS analyses were performed as previously described (Oleszek et al. 1988).

Model system of phenolics and ODP. Epicatechin, procyanidin B2 and C1, five quercetine and two phloretine glycosides and chlorogenic acid were individually subjected to ODP. Substrate solutions of 0.2 mM in acetate buffer at pH 5.5 were prepared for each phenolic compound. To 10 cm³ substrate solution 0.15 cm³ (1000 AU) of ODP (Sigma Chem. Comp. St. Louis, MO) solution was added and changes in color were measured at 420 nm using a spectrophotometer (Helwett-Packard 8452A) equipped with a diode array detector and a series 300 computer. For each test a sample without enzyme added was used as a reference.

Synergistic effect of quercetine and phloretine glycosides in the browning reactions. Effects of quercetine and phloretine glycosides on the rate of browning of various substrates: chlorogenic acid, epicatechin, B2 and C1 (0.2 and 0.4 mM solutions) were studied in a model system. For this study, 5 cm³ of 0.4 mM of glycoside in acetate buffer (pH 5.5) solution was mixed with 5 cm³ of 0.4 mM substrate solution and then 0.15 cm³ of enzyme were added. The rate of browning was measured at 420 nm as above during a 3 h period.

RESULTS AND DISCUSSION

The phenolic acid fraction of the apple skin extract showed a single spot when analysed by TLC. The R_f of this spot was identical to that of the chlorogenic acid standard. However, HPLC analysis on a C_{18} column using

a linear gradient of 40% acetonitrile in 5% acetic acid as a mobile phase (Jaworski and Lee 1987) revealed two peaks with retention times (R_t) of 15.3 and 24.4 min. The first R_t was consistent with that of the chlorogenic acid standard. The second peak was not identified but its peak area was only 3% of that of chlorogenic acid. FAB-MS of the first compound produced the spectrum with following ions: m/z (rel.int.) (positive ion mode) — 1063(1) $[2MH]^+$, 731(4) $[3MNa]^+$, 709(8) $[2MH]^+$, 377(43) $[MNa]^+$, 355(100) $[MH]^+$, 193(43) $[Quinic\ acid + H]^+$; (negative ion mode) — 1061(1) $[3M-H]^-$, 707(20) $[2M-H]^-$, 353(100) $[M-H]^-$, 191(67) $[Quinic\ acid - H]^-$. This spectrum was in general agreement with the one reported before (Sakushima et al. 1985). In the spectrum, however, strong ions at 193 in the positive and 191 in the negative ion modes were interpreted as $[Quinic\ acid + H]^+$ and $[Quinic\ acid - H]^-$, respectively. This does not fully confirm the scheme of FAB-MS fragmentation of chlorogenic acid as proposed by Sakushima et al. (1985), who showed that the negative charge was generated on the quinic acid fragment and the positive charge on the caffeic acid part. Our spectrum shows both positive and negative charges of the quinic acid fragment but not the caffeic acid ion.

The separation of a non-acidic phenolic group containing catechins and procyanidins on Sephadex LH20 yielded four fractions, three of which were identified. The first fraction (80 mg), when analysed by HPLC, showed two peaks with retention times of 12.5 and 20.1 min, corresponding to catechin and epicatechin, respectively, and the peak ratio was 4:96. The TLC showed only one spot with an R_f value identical to that of the epicatechin standard. The HPLC of the second fraction (320) revealed two peaks with R_t of 9.1 and 15.1 min with a peak area ratio 4.5:95.5. FAB-MS produced the spectrum with a pronounced ion at m/z (rel.int.) (positive ion mode) — 601(33) $[MNa]^+$, 579(100) $[MN]^+$, 427(57), 411(57), 391(36); (negative ion mode) — 1155(13) $[2M-H]^-$, 577(100) $[M-H]^-$, 425(35). The confirmed structure was procyanidin B2 as reported previously (Self et al. 1986). It seems that the small peak at R_t 9.1 can be regarded as an isomer of a procyanidin dimer which cannot be distinguished from B2 by FAB-MS measurements. The presence of a quite strong ion at 1155 in the negative ion mode spectrum can be interpreted as $[2M-H]^-$ rather than as an indicator of the presence of a procyanidin tetramer. Thus procyanidin B2 was proved to be the major component of apple skin dimers.

The HPLC of the third fraction (150 mg) contained three peaks: R_t 9.1, 19.8 and 29.5, with the ratio of peak areas, 2.5:90.5:7.5. This fraction gave the following FAB-MS spectrum: m/z (rel.int.) (positive ion mode) — 889(40) $[MNa]^+$, 867(67) $[MH]^+$, 715(20), 601(29), 579(100); (negative ion mode) — 865(100) $[M-H]^-$, 713(33), 577(69). These are characteristic of procyanidin C1 as reported previously (Self et al. 1986). No higher ions were registered which proves that a procyanidin tetramer was not among the trace compounds in this

fraction. Strong ions at 579(100) in positive and 577(69) in negative ion modes may originate from the trimer, but as was reported before (Self et al. 1986) they usually do not exceed 10% of the parent peak intensity. This high intensity suggests that the sample was contaminated with some procyanidins dimers which, as shown above for procyanidin B2, contributed strong molecular ions. It was concluded thus that separated procyanidin trimer fraction consisted of trimer C1 as a dominant compound and some dimers which were present in trace amounts.

The compounds identified in this study as well as those reported before (Oleszek et al. 1988) were studied for their participation in oxidative reactions and color development. Catechins, procyanidin and phloretine glycoside solutions resulted in a wide range of color development when individually subjected to ODP (Fig. 1). The absorbance readings at 420 nm showed that catechin and epicatechin turned brown rapidly, whereas procyanidin B2 and C1 reacted very slowly and needed at least 48 h to develop the same color intensity as by catechins after 3 h. These data generally support the previous reports that flavan monomers were the most readily oxidizable phenolic compounds and the browning rates of flavan polymers e.g. B2 and C1 were much lower than monomers. However in the terms of prolonged reaction time, these polymers seem to be also important when the reaction time was extended, since their full color development after 48 h was equal to that developed by monomers in a short time.

Fig. 1. The rate of browning of: catechin (1), epicatechin (2), phloretine xyloglucoside (3), phloridzin (4), chlorogenic acid (5), procyanidin C1 (6) and procyanidin B2 (7). All substrates at the concentration of 0.2 mM and ODP activity at 1000 AU/10 cm³ of solution

The browning rate of chlorogenic acid at the beginning was very close to that of catechin and epicatechin, but reached a plateau very quickly and after 48 hr of the reaction, the color intensity was about three times lower than those of procyanidins. Thus, chlorogenic acid alone seems to be a less significant factor in color development than flavans. The phloretine glycoside solutions turned brown slowly but after 3 h their absorbances were intermediate between those of catechins and procyanidins, with phloretine xyloglucoside being more readily oxidized than phloridzin. After 24 h of reaction, however, the absorbance values of the phloretine glycosides were equal to each other and reached a value of 1.2, the highest of all compounds tested. This finding does not support the previous reports suggesting that phloridzin was a poor ODP substrate (Durkee and Poapst 1965). Our results clearly demonstrate that both phloretine glycosides were good ODP substrates. Phloretine xyloglucoside, whose contribution to browning was not previously reported, was more readily oxidised than phloridzin.

None of the apple skin flavonoid glycosides reacted with ODP when treated individually. This finding is in good agreement with previous reports (Roberts and Wood 1951, Baruach and Swain 1959) on the oxidation of some flavonoid compounds with potato or tea oxidases, which showed that some flavonoid aglycones can be regarded as ODP substrates but glucosidation totally prevented the reaction.

It was also previously reported that some flavonoids may display an antioxidant activity (Abdel-Rahman 1985, Araujo and Pratt 1985). Thus we deemed it of interest to study the role of apple quercetine and phloretine glycosides as antioxidants in model systems. When five quercetine glycosides were each added at 0.2 mM concentration to a 0.2 mM solution of epicatechin, the browning rates of the resulting mixtures showed some changes after 1 h as compared to those of epicatechin as a sole substrate (Fig. 2). Quercetine galactoside was the only one of the five which decreased the absorbance, whereas quercetine glucoside, xyloside, arabinoside and rhamnoside slightly increased. However, the quercetine glycoside/epicatechin mixture had a pronounced effect on the general spectra (Fig. 3); the maximum absorption (0.75 units) of epicatechin was at 376 nm after 3 h, but the maximum absorption in the spectra of the mixtures of epicatechin/quercetine glycosides was shifted to a higher wavelength. The degree of these shifts was characteristic for every individual glycoside; absorption maxima were at 398 nm (absorption 0.35), 410 nm (0.53), 406 nm (0.54), 402 nm (0.51) and 402 nm (0.57) for quercetine galactoside, glucoside, xyloside, arabinoside and rhamnoside, respectively. Since the structural differences of particular glycosides lay only in their sugar substitution, it was unclear why they behaved differently. Partial explanation of this phenomenon can be found in the close examination of the general spectra of the mixtures as shown in Fig. 3. The absorption maxima of

Fig. 2. The rate of browning of epicatechin (5) and its mixture with quercetine glycosides: rhamnoside (1), xyloside (2), glucoside (3), arabinoside (4) and galactoside (6). All compounds at the concentration of 0.2 mM

Fig. 3. The general spectra of epicatechin (1) and its mixture with quercetine galactoside (2) and quercetine xyloside (3), after oxidation with ODP for 3 h

flavonoid/epicatechin mixture showed shifts to higher wavelengths, different for particular glycosides. Since the epicatechin/quercetine galactoside spectrum showed the smallest shift, its absorbance at 420 nm is lower than that of epicatechin, and this may cause it to be misinterpreted as an inhibitor. Thus, it is clear that flavonoid glycosides, when oxidised in the mixture with the other

ODP substrates, may participate in synergistic color development, therefore their role in the browning reactions should not be neglected.

A significant increase in absorbance was observed in mixtures of epicatechin/phloretine glycosides and ODP. Both phloridzin and phloretine xyloglucoside enhanced the rate of browning drastically. Fig. 4 shows that both the mixture of epicatechin/phloridzin and epicatechin/phloretine xyloglucoside at concentrations of 0.2 mM after 3 h oxidation gave absorbance values of 1.25 and 1.21, respectively. This value was several fold higher than that found for other phenolics (both at 0.2 and 0.4 mM). Changes were also observed in the visible spectra of oxidised mixtures. Fig. 5 compares the spectra of individual phloridzin and epicatechin and their mixtures when oxidised by ODP. The reaction of the mixture produced the highest absorbance in a wide range of wavelengths in comparison to both compounds treated separately. The same shape of spectrum was obtained for phloretine xyloglucoside and its mixture with epicatechin.

Fig. 4. The rate of browning of: 1 — epicatechin (0.2 mM), 1a — epicatechin (0.4 mM), 1/2 — epicatechin/phloridzin (0.2 mM each), 1/3 — epicatechin/phloretine xyloglucoside (0.2 mM each), 2 — phloridzin (0.2 mM), 2a — phloridzin (0.4 mM), 3 — phloretine xyloglucoside (0.2 mM)

Similar results were obtained when chlorogenic acid and procyanidins B2 and C1 were used as substrates in the mixture with phloridzin (Fig. 6). The browning rate of chlorogenic acid/phloridzin mixture (both at 0.2 mM concentration) was several times higher than that of chlorogenic acid alone (0.4 mM) and was very close to the browning rate of a epicatechin/phloridzin (0.2 mM each) mixture. The addition of phloridzin at 0.2 mM concentrations into procyanidin B2 and C1 (0.2 mM) solutions also resulted in a large increase

Fig. 5. The general spectra of epicatechin (2), phloridzin (3) and their mixture (1) when oxidized with ODP for 3 h

Fig. 6. The rate of browning of mixtures: phloridzin/chlorogenic acid (1), phloridzin/procyanidin C1 (2), phloridzin/procyanidin B2 (3) (0.2 mM each); and chlorogenic acid (4), procyanidin C1 (5), procyanidin B2 (6) (0.4 mM each)

in their browning rates. Although these rates were lower than those of epicatechin/phloridzin and chlorogenic acid/phloridzin mixtures, they were much higher than procyanidin B2 or C1 alone at a 0.4 mM concentration.

The synergistic effect of phloretine glycosides on the browning reaction is reported here for the first time. We believe that this synergistic effect is a very

important factor in determining browning of apple products since those glycosides are present in significant amounts in skin and flesh of apples (Oleszek et al. 1988). In addition these findings clearly show that the rate of browning in apple products cannot be regarded as a linear function of the phenolic content, since the rate of browning is not additive of particular ODP substrates. This may explain the difficulties in finding a linear correlation between total phenolic contents and the rate of browning of apple products as reported previously (Vamos-Vigyazo and Gajzago 1978, Coseteng and Lee 1987). It should be emphasized also that the present studies were performed in model systems. Therefore, a closer study in complex systems including many other factors that influence the browning reactions should be carried out in order to prevent browning in our foods.

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Fenole jabłek i ich udział w reakcjach ciemnienia enzymatycznego

Streszczenie

Ze skórki jabłek wyizolowano kwas chlorogenowy, epikatechinę i procyanidynę B2 i C1. Związki te, jak również glikozydy kwercetyny i fłoretyny, również wyizolowane z jabłek, badano pojedynczo i w mieszaninach pod kątem ich udziału w reakcjach ciemnienia enzymatycznego. Stwierdzono, że najlepszymi substratami dla oksydazy o-difenolowej (ODP) są proste flawany, jeśli utleniane są pojedynczo. Procyanidyny i kwas chlorogenowy ulegały utlenianiu enzymatycznemu znacznie wolniej. Wskazano na udział glikozydów kwercetyny i synergistyczny wpływ glikozydów fłoretyny na enzymatyczne utlenianie kwasu chlorogenowego i flawanów.